



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : C12Q 1/68	A2	(11) International Publication Number: WO 97/32042 (43) International Publication Date: 4 September 1997 (04.09.97)
<p>(21) International Application Number: PCT/GB97/00577</p> <p>(22) International Filing Date: 3 March 1997 (03.03.97)</p> <p>(30) Priority Data: 9604480.5 1 March 1996 (01.03.96) GB 9605598.3 16 March 1996 (16.03.96) GB</p> <p>(71) Applicant (for all designated States except US): THE UNIVERSITY COURT OF THE UNIVERSITY OF DUNDEE [GB/GB]; Tower Building, Dundee DD1 4HN (GB).</p> <p>(72) Inventor; and (75) Inventor/Applicant (for US only): BURCHELL, Brian [GB/GB]; 8 Dougall Street, Tayport, Fife DD6 9 JB (GB).</p> <p>(74) Agent: MURGITROYD & COMPANY; 373 Scotland Street, Glasgow G5 8QA (GB).</p>	<p>(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).</p> <p>Published Without international search report and to be republished upon receipt of that report.</p>	
<p>(54) Title: DRUG TRIAL ASSAY SYSTEM</p> <p>(57) Abstract</p> <p>The invention provides a method for improving the efficacy of drug trials, the method comprising the step of screening samples from potential participants for the genetic basis of Gilbert's Syndrome and eliminating or including potential participants in a drug trial in the knowledge of them possessing or not possessing the genetic basis of Gilbert's Syndrome.</p>		

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1 "Drug Trial Assay System"

2

3 The present invention relates to drug trials, usually
4 carried out for or on behalf of pharmaceutical
5 companies. More particularly the invention relates to
6 a method for improving the efficacy of drug trials.

7

8 In the different stages of drug trials, regulatory
9 authorities in different European countries and the FDA
10 in the USA require extensive data to be provided in
11 order to approve use of the drugs.

12

13 It is important that as much information as possible is
14 available in relation to all participants who take part
15 in drug trials, from volunteers who take part in phase
16 1 trials to patients involved in stage 3 clinical
17 trials.

18

19 In particular, if certain individuals or groups of
20 individuals have severe or abnormal reactions to drug
21 administration, further studies involving that drug
22 will be in jeopardy unless the reason for the reaction
23 is realised.

24

25 The knowledge of pharmacogenetics can play an important

1 role in understanding the impact of drug metabolism on
2 pharmacokinetics, role of receptor variants in drug
3 response and in the selection of patient populations
4 for clinical studies.

5
6 Considerable effort has been expended in attempting to
7 identify the pharmacogenetic basis of idiosyncratic
8 adverse drug reactions, particularly hypersensitivity
9 reactions. While there is clear evidence for
10 pharmacogenetic influence on susceptibility to
11 hypersensitivity reactions, necessary and sufficient
12 pharmacogenetic defects have not been identified.

13
14 The clinical implications of genetic polymorphism in
15 drug metabolism have been studied extensively (See
16 Tucker GT (1994) Journal Pharmacology 46 pages 417-
17 424).

18
19 Gilbert's Syndrome (GS) is a benign unconjugated
20 hyperbilirubinaemia occurring in the absence of
21 structural liver disease and overt haemolysis and
22 characterized by episodes of mild intermittent
23 jaundice. It is part of a spectrum of familial
24 unconjugated hyperbilirubinaemias including the more
25 severe Crigler-Najjar (CN) syndromes (types 1 and 2).
26 GS is the most common inherited disorder of hepatic
27 bilirubin metabolism occurring in 2-12% of the
28 population and is often detected in adulthood through
29 routine screening blood tests or the fasting associated
30 with surgery/intercurrent illness which unmasks the
31 hyperbilirubinaemia^{1,3}. The most consistent feature in
32 GS is a deficiency in bilirubin glucuronidation but
33 altered metabolism of drugs has also been reported^{3,5}.
34 Altered rates of bilirubin production, hepatic haem
35 production and altered hepatic uptake of bilirubin have
36 been reported in some GS patients².

1 Due to the benign nature of the syndrome and its
2 prevalence in the population it may be more appropriate
3 to consider GS as a normal genetic variant² exhibiting a
4 reduced bilirubin glucuronidation capacity (which in
5 certain situations such as fasting, illness or
6 administration of drugs) could precipitate jaundice.

7
8 In drug trials where high levels of serum total
9 bilirubin is detected for certain individuals, it is
10 not clear whether this is because the individuals have
11 Gilbert's Syndrome or if it because of an effect of the
12 drug. Whereas presently, results are explained merely
13 by saying that the individuals have Gilbert's Syndrome,
14 it is suspected that in the future, it will be
15 necessary to prove this fact.

16
17 Where a jaundiced phenotype is apparent after
18 volunteers have been accepted for a trial and have been
19 subjected to five days of a strict diet, no alcohol and
20 no smoking, the jaundiced appearance giving an
21 indication that the individuals have Gilbert's
22 Syndrome, may cause them to be ruled out of the trials.
23 Therefore, where approximately 250 individuals would be
24 required for phase 1 trials and about 6000 patients for
25 phase 3 trials, unnecessary time and effort would have
26 been spent during the first 5 days of these trials and
27 individuals having Gilbert's Syndrome may be ill
28 effected.

29
30 The present invention aims to provide a method of
31 improving the efficacy of drug trials in view of the
32 problems mentioned above.

33
34 According to the present invention there is provided a
35 method for improving the efficacy of drug trials, the
36 method comprising the step of screening samples from

1 individuals for the genetic basis of Gilbert's
2 Syndrome.

3
4 In a preferred embodiment of the invention the method
5 comprises the steps taking a sample from each potential
6 participant in a drug trial, screening the samples for
7 the genetic basis of Gilbert's Syndrome, identifying
8 participants having the genetic basis of Gilbert's
9 Syndrome.

10

11 The sample may comprise blood, a buccal smear or any
12 other sample containing DNA from the individual to be
13 tested.

14

15 In one embodiment the method comprises the further step
16 of eliminating participants having the genetic basis of
17 Gilbert's Syndrome from the drug trial.

18

19 In an alternative embodiment, the method can comprise
20 the further step of selecting participants having the
21 genetic basis of Gilbert's syndrome and eliminating
22 others from the drug trial.

23

24 In a further alternative the results of the drug trials
25 can be interpreted in the knowledge that certain
26 participants have Gilbert's Syndrome.

27

28 Preferably the method comprises the steps of isolating
29 DNA from each sample, amplifying the DNA in a region
30 indicating the genetic basis of Gilbert's Syndrome,
31 isolating amplified DNA fragments by gel
32 electrophoresis and identifying individuals having the
33 genetic basis of Gilbert's disease.

34

35 Preferably the DNA is amplified using the polymerase
36 chain reaction (PCR) using a radioactively labelled

1 pair of nucleotide primers.

2

3 The primers are designed to prime the amplification
4 reaction at either side of an area of the genome known

5

6 to be associated with Gilbert's Syndrome.

7

8 Preferably the DNA region indicating the genetic basis
9 of Gilbert's Syndrome is the gene encoding UDP-
10 glucuronosyltransferase (UGT).

11

12 By gene is meant, the non coding and coding regions and
13 the upstream and downstream noncoding regions.

14

15 In a preferred embodiment the DNA to be amplified is in
16 an upstream promoter region of the UGT1*1 exon1.

17

18 Most preferably the DNA to be amplified includes the
19 region between -35 and -55 nucleotides at the 5' end of
20 UGT1*1 exon.

21

22 According to the invention there are provided suitable
23 primers for use in a PCR reaction including primer
24 pairs;

25

26 A/B (A, 5'-AAGTGAAGTCCCTGCTACCTT-3',
27 B, 5'-CCACTGGGATCAACAGTATCT-3') or
28 C/D (C, 5'-GTCACGTGACACAGTCAAAC-3';
29 D 5'-TTTGCTCCTGCCAGAGGTT-3')

30

31 The invention further comprises a kit for screening
32 individuals for participation in drug trials, the kit
33 comprising primers for amplifying DNA in a region of
34 the genome indicating the genetic basis of Gilbert's
35 Syndrome.

36

1 Using primer sequences as described herein, DNA can be
2 amplified and analysed using among others any of the
3 following protocols;

4
5 Protocol 1 Radioactive method

- 6
7 1. Extract DNA from Buccal Cells or 3ml Blood.
8
9
10 2. Choose primers from either side of the "TATA" box
11 region of UGT1*1 exon1 regulatory sequence.
12 Freshly end label one primer with [γ 32 α]-ATP (40
13 min).
14
15 3. Amplifying a small region up to 100 bp in length
16 by PCR (2h).
17
18 4. Apply to 6% PAG denaturing gel (preparation,
19 loading, run time, 4h).
20
21 5. Expose (-70°C) wet gel to autoradiographic film
22 (15 min).
23

24 This method takes about 7h to complete. Polymorphisms
25 only observed in TATA box non coding region todate.

26
27 Protocol 2

28 Alternative Radioactive Method: Solid Phase
29 Minisequencing

- 30
31 1. Extract DNA (as above)
32
33 2. Prepare primers biotinylating one
34
35 3. Amplify DNA by PCR using primers
36

1 4. Captive biotinylated PCR products on streptavidin
2 coated support and deactive.

3
4 5. Carry out primer extension reaction sequencing.

5
6 Protocol 3

7 Non-Radioactive Methods:

8
9 (a) Analysis by Single Strand Conformational
10 Polymorphism (SSCP)

11 1. Extract DNA (as above).

12
13 2. Choose primers either side of the TATA Box.

14
15 3. Amplify a small region up to 100 bp in length by
16 PCR (2H).

17 4. Denature and place on ice (15 min).

18
19 5. Load onto a non-denaturing PAG gel,
20 (preparation/load/run time, 4h).

21
22 6. Stain with Ethidium bromide or silver nitrate (30
23 mm).

24
25 This method still takes about 7h to complete, but is
26 potentially slightly cheaper since there is no
27 radioactivity or autoradiography.

28
29 This method could be done on an automated DNA sequencer
30 from stage 5, if primers are tagged with chromophores
31 in PCR stages 2 and 3. Result would then be read
32 automatically.

33
34 (b) Oligonucleotide Assay Hybridization

35
36 1. Extract DNA (as above).

- 1 2. Choose primers and amplify DNA by PCR up to 100 bp
2 in length.
- 3
- 4 3. Apply DNA to plastic grids.
- 5
- 6 4. Screen bound DNA samples with specific DNA probes
7 for TA₅, TA₆, TA₇ tagged with different
8 coloured/fluorescent chromophores.
- 9
- 10 5. Read output automatically for experimental
11 protocols.
- 12

13 References

- 14
- 15 Monaghan G et al. Lancet (1996) 347 578-581.
- 16
- 17 "Detection of polymorphisms of human DNA by gel
18 electrophoresis or single-strand conformational
19 polymorphisms". Orita M et al. Proc Natl Acad Sci
20 (USA) (1989) 86 2766-2700.
- 21
- 22 "Assays of complementary oligonucleotides for analysing
23 Hybridization behaviour of Nucleic Acids". Southern E
24 M. Nuc Acids Res (1994) 22 1368-1373.

1 The basis of the invention is illustrated in the
2 following example with reference to the accompanying
3 figures wherein:

4
5 Figure 1 illustrates genotypes at the TATA box sequence
6 upstream of the UGT1*1 exon 1 determined by direct
7 sequencing and radioactive PCR.

8
9 Figure 2 illustrates serum total bilirubin ($\mu\text{mol/l}$)
10 plotted against UGT1*1 exon 1 genotype.

11
12 Figure 3 illustrates segregation of the 7/7 genotype
13 with elevated serum total bilirubin concentration in a
14 family with GS.

15
16 Figure 4 illustrates the 5' sequence of the UGT1*1 exon
17 1 and the position of the primers with respect to the
18 UGT gene.

19
20 Example

21
22 We have examined the variation in the serum total
23 bilirubin (STB) concentration in a representative group
24 of the Eastern Scottish population (drug-free, alcohol-
25 free non-smokers) in relation to genotype at the UDP-
26 glucuronosyltransferase subfamily 1 (UGT1) locus.
27 Subjects with the 7/7 genotype in this population have
28 a significantly higher STB than those with 6/7 or 6/6
29 genotypes. Of 14 control subjects who underwent a 24
30 hour fast to establish whether they had Gilbert
31 Syndrome (GS), only 7/77 subjects had GS. In addition,
32 one confirmed GS patient, two recurrent jaundice
33 patients and 9 clinically diagnosed GS patients had the
34 7/7 genotype. Segregation of the 7/7 genotype with
35 elevated STB concentration has also been demonstrated
36 in a family of 4 Gilbert members. This incidence of

1 the 7/7 genotype in the population is 10-13%. Here, we
2 demonstrate a correlation between variation in the
3 human STB concentration and genotype at a TATA sequence
4 upstream of the UGT1*1 exon 1 and that the 7/7 genotype
5 is diagnostic for GS.

6
7 The inheritance of GS has been described as autosomal
8 dominant or autosomal dominant with incomplete
9 penetrance based on biochemical analysis⁶. More recent
10 reports have suggested that the mildly affected
11 (Gilbert) members of families in which CN type 2 (CN-2)
12 occurs are heterozygous for mutations in the UDI³-
13 glucuronosyltransferase subfamily 1 (UGT1) gene which
14 cause CN-2 in the homozygous state. The inheritance of
15 GS in these families is autosomal dominant while CN-2
16 is autosomal recessive⁷⁻¹¹. However, the incidence of
17 CN-2 in the population is very rare and the frequency
18 of alleles causing CN-2 would not be sufficient to
19 explain the population incidence of GS.

20
21 An abstract by Bosma et al¹² suggested a correlation
22 between homozygosity for a 2bp insertion in the TATA
23 box upstream of UGT1*1 exon 1 and GS (no mutations were
24 found in the coding sequence of the UGT1*1 gene). In
25 this report we demonstrate that the primary genetic
26 factor contributing to the variation in the serum total
27 bilirubin (STB) concentration in the Eastern Scottish
28 population is the sequence variation reported by Bosma
29 et al¹². In addition, we show that the 7/77 genotype is
30 associated with GS and occurs in 10-13% of the
31 population.

32

33 Methods

34 Patients and Controls

35 Whole blood (3ml) was collected into EDTA(K3)

36 Vacutainer tubes (Becton Dickinson) from one confirmed

1 male Gilbert patient (diagnosed following a 48 hour
2 restricted diet¹³), two female patients with recurrent
3 jaundice/associated elevated STB (29-42 $\mu\text{mol/l}$) and 9
4 (1 female, 8 male) clinically diagnosed GS subjects
5 (persistent elevation of the STB amidst normal liver
6 function tests.) The patients were aged 22-45 years.

7
8 77 non-smoking residents selected at random from the
9 Tayside/Fife region of Scotland (39 females aged 19-58
10 years, mean 32.41 ± 10.94 ; 38 males aged 23-57, means
11 35.58 ± 9.04) participated in this study. Whole blood
12 (9ml) was collected 8-10am into EDTA(K3) Vacutainer
13 tubes (Becton Dickinson) for DNA extraction and SST
14 Vacutainer tubes (Becton Dickinson) for biochemical
15 investigations. The subjects had not taken any
16 medication or alcohol in the previous 5-7 days and had
17 fasted overnight (12 hours). 14 controls subsequently
18 underwent further biochemical tests (following a 3 day
19 abstinence from alcohol) before and after a 24 hour
20 400-calorie diet¹⁴ to determine if they had GS. All
21 patients/controls were fully informed of the study and
22 gave consent for their blood to be used in this study.

23 24 Biochemistry and DNA Extraction

25
26 The following biochemical tests were performed on
27 control blood samples; alanine aminostransferase,
28 albumin, alkaline phosphatase, amylase, STB,
29 cholesterol, creatinine, creatine kinase, free
30 thyroxine, gamma-glutamyl-transferase, glucose, HDL-
31 cholesterol, HDL-cholesterol/total cholesterol, iron,
32 lactate dehydrogenase, percentage of saturated
33 transferrin (PSAT), proteins, serum angiotensin
34 converting enzyme, thyroid stimulating hormone,
35 transferrin, triglycerides, urate, urea. 14 controls
36 also had pre- and post-fasting (24 hour) alanine

1 aminostransferase, albumin, alkaline phosphatase, STB
2 and urate measured. DNA was prepared using the Nucleon
3 II Genomic DNA Extraction Kit (Scotlab) according to
4 manufacturer's instructions.

5

6 Genotyping

7

8 Polymerase Chain Reaction

9

10 Primer pairs A/B (A, 5'-AAGTGAAGTCCCTGCTACCTT-3'; B,
11 5'-CCACTGGGATCAACAGTATCT-3') or C/D (C, 5'-
12 GTCACGTGACACAGTCAAAC-3'; D, 5'-TTTGCTCCTGCCAGAGGTT-3')
13 flanking the TATA box sequence upstream of the UGTI*1
14 exon 1 were used to amplify fragments of 253-255bp and
15 98-100bp, respectively. Amplifications (50µl) were
16 performed in 0.2mM of each deoxynucleoside triphosphate
17 (dATP, dCTP, dGTP, dTTP), 50mM KCl, 10mM Tris.HCl (pH
18 9.0 at 25°C), 0.1% Triton X-100, 1.5mM MgCl₂, 0.25µM of
19 each primer, 1 Unit of Taq Polymerase (Promega) and
20 human DNA (0.25-0.5µg). The polymerase chain reaction
21 (PCR) conditions using the Perkin-Elmer Cetus DNA
22 Thermal Cycler were: 95°C 5 min followed by 30 cycles
23 of 95° 30 sec, 58°C 40 sec, 72°C 40 sec.

24

25 Direct Sequencing

26

27 Amplification was confirmed prior to direct sequencing
28 by agarose gel electrophoresis. Sequencing was
29 performed using [α -³⁵S]-dATP (NEN Dupont) with the USB
30 Sequenase™ PCR Product Sequencing Kit according to
31 manufacturer's instructions. Sequenced products were
32 resolved on 6% denaturing polyacrylamide gels. The
33 dried gels were exposed overnight to autoradiographic
34 film prior to developing.

35

36 Radioactive PCR

1 Amplification was performed as above using primer pair
2 C/D except that 2.5 pmol of primer C was radioactively
3 5' end-labelled with 2.5 μ Ci of [γ - 32 P]-ATP (NEN Dupont)
4 prior to amplification. Products were resolved on 6%
5 denaturing polyacrylamide gels and the wet gels exposed
6 to autoradiographic film (-70°C 15 min) and the
7 autoradiographs developed.

8

9 Statistics

10

11 A t-test was used to determine if there was a
12 significant age difference between males and females.
13 χ^2 analysis was used to assess any difference in the
14 distribution of the 6/6, 6/7 and 7/7 genotypes in males
15 and females and also to determine if the 7/7 subjects
16 from the 24 hour fasted group had STB elevated into the
17 range diagnostic for GS¹⁴. An analysis of variance was
18 performed to compare mean STB in males and females
19 within each genotype group. A non-parametric test, the
20 Mann-Whitney U-Wilcoxon Rank Sum W Test was used to
21 determine whether there was a significant difference in
22 mean STB between males and females (irrespective of
23 genotype). Correlations and significance tests were
24 performed for STB versus PSAT and STB versus iron. A
25 probability (p) of ≤ 0.05 was accepted as significant.

26

27 Results

28

29 In Figure 1 a photographic representation of the sense
30 DNA sequences obtained by PCR/direct sequencing of DNA
31 samples having the genotypes 6/6, 6/7 and 7/7 is shown.
32 The common allele, (TA)₆TAA, is denoted by "6" while the
33 rarer allele, (TA)₇TAA, is denoted by "7". Below each
34 sequence is an overexposed photographic representation
35 of the 98 to 100bp resolved fragments amplified using
36 primer pair C/D which flank the TATA sequence upstream

1 of the UGT1*1 exon 1. The additional fragments of 99
2 and 101 bases are thought to be artifacts of the PCR
3 process where there is non specified addition of an
4 extra nucleotide to the 3' end of the amplified
5 product²¹. Figures 1b illustrates results after testing
6 a range of unknown individuals.

7
8 In Figure 2 males (M) and females (F) are plotted
9 separately. Each circle/square represents the result
10 of a single control subject. The squares indicate the
11 14 controls who also underwent the 24 hour restricted
12 diet (see Methods). The filled circles/squares
13 represent those who had a lower than normal PSAT (\leq
14 22%) while the half-tone circles represent those who
15 had a higher than normal PSAT (\geq 55%). The mean STB
16 concentrations (indicated by the horizontal lines) for
17 males were 13.24 ± 3.88 (6/6), 13.94 ± 6.1 (6/7)
18 including control h or 12.69 ± 3.34 excluding control
19 h, 29 ± 14.45 (7/7) and for females were 9 ± 3.62
20 (6/6), 12.2 ± 3.53 (6/7), 21.6 ± 7.8 (7/7). The
21 encircled result is from control h (discussed in the
22 text).

23
24 In Figure 3 males and females are represented by
25 squares and circles, respectively. Filled and half-
26 filled circles/squares indicate the genotypes 7/7 and
27 6/7, respectively. The numbers in parentheses below
28 each member of the pedigree are the STB concentrations
29 measured after a 15 hour fast and 7 day abstinence from
30 alcohol. All family members were non smokers who were
31 not taking any medication when the biochemical tests
32 were performed. Elevated STB are underlined.
33 Individual members of each generation (I or II) are
34 denoted by the numbers 1-4 above each circle/square.
35 Generation III have not yet been tested.
36

1 There was no significant age difference between males
2 and females ($t = -1.38$, $p = 0.17$). Genotypes were
3 determined initially by amplification/sequencing and
4 later by the radioactive PCR approach. Individuals
5 homozygous for the common allele, heterozygous or
6 homozygous for the rarer allele have the genotypes 6/6,
7 6/7 and 7/7, respectively. 12 DNA samples (2 of 6/6, 3
8 of 6/7 and 4 of 7/7) were analysed by both methods and
9 genotype results were identical (see Figure 1).
10
11 Genotype frequencies in male controls were 6/6 (44.74%,
12 6/7 (44.74%), 7/7 (10.52%) and in female controls were
13 6/6 (35.9%), 6/7 (51.3%), 7/7 (12.8%). There was no
14 significant difference between the genotype proportions
15 in the two groups ($\chi^2 = 0.6$ at 2 df, $p = 0.7$). Control
16 h (encircled in Figure 2) had a STB which was 2.4 SD
17 above the mean STB for that group (mean calculated
18 including control h). The results for control h were
19 repeatable and he is currently being investigated to
20 exclude haemochromatosis. Comparison of mean STB in
21 males and females revealed that females have a
22 significantly lower concentration than males ($p = 0.031$
23 including control h; $p = 0.0458$ excluding control h).
24 There was a strong correlation between genotype and
25 mean STB concentration within the control group ($p <$
26 0.001) irrespective of whether control h was included
27 and there was a significant difference in mean STB
28 between males and females of the same genotype ($p <$
29 0.05) irrespective of whether control h was included
30 (see Figure 2). All patients studied had the 7/77
31 genotype.
32
33 Correlations between STB/PSAT ($r = 0.4113$, $p =$
34 0.001) (see Figure 2) and STB/iron females ($p = 0.001$)
35 than males ($p = 0.01$) but when control h is excluded
36 there was no significant correlation in males.

1 The STB concentrations of control who underwent the 24
2 hour restricted diet (see Methods) are shown in Table
3 1. The normal fasting response is a small rise in the
4 base-line STB (not exceeding a final concentration of
5 $25\mu\text{mol/l}$) most of which is unconjugated while GS
6 patients have a lone biochemical feature a raised STB
7 ($>25\mu\text{mol/l}$ but $<50\mu\text{mol/l}$) most of which is
8 unconjugated¹⁴. The 6/6 and 6/7 controls had post-
9 fasting STB of $\leq 23\mu\text{mol/l}$ while all 7/77 controls were
10 $\geq 31\mu\text{mol/l}$. Other liver function tests were within
11 acceptable ranges for the age and sex of the subjects.
12 The 7/77 genotype correlates with a fasted STB (24
13 hour) within the range diagnostic for GS¹⁴ ($p <$
14 0.01) (see Table 1). In addition, the 7/7 genotype
15 segregates with elevated STB concentration in a family
16 with 4 GS members (Figures 3).

17
18 Table 1 shows a comparison of the UGT1*1 exon 1
19 genotype with elevation in the serum total bilirubin
20 after a 24 hour 400-calorie restricted diet¹⁴.

21
22 An elevation of the fasting STB to a final
23 concentration in the range $25-50\mu\text{mol/l}$ is considered to
24 be diagnostic for GS¹⁴. The 7/7 subject denoted by *
25 has a fasting and non-fasting STB of $> 50\mu\text{mol/l}$ but
26 this value is within a range considered by others to
27 conform to a diagnosis of GS⁷⁻¹¹.

Table 1

Genotype	Sex	24 hour fast		Fasting bilirubin >25 & <50 μ mol/l
		Before	After	
6/6	M	8	17	NO
	M	9	19	NO
	M	12	15	NO
6/7	F	8	17	NO
	F	9	13	NO
	F	11	12	NO
	F	12	17	NO
	M	8	10	NO
	M	15	23	NO
	M	17	18	NO
7/7	F	9	34	YES
	F	12	34	YES
	M	19	31	YES
	M	62	96	NO*

1 Discussion

2

3 A few recent reports claim to have identified the
 4 genetic cause of GS¹⁰⁻¹². Clinical diagnosis of GS is
 5 often based on a consistent mildly elevated non-fasting
 6 STB (>17 μ mol/l) as the sole abnormal liver function
 7 test, intermittent jaundice or both. The diagnosis can
 8 be confirmed by elevation of the STB to 25-50 μ mol/l
 9 after a 24 hour 400-calorie diet¹⁴ or by elevation of
 10 the unconjugated bilirubin by > 90% within 48 hours of
 11 commencing a 400 calorie diet¹³.

12

13 Sato's research group recently reported the occurrence
 14 of 7 different heterozygous missense mutations in
 15 unrelated Gilbert patients (most of the mutations have
 16 been found in the homozygous state in affected members
 17 of CN families), however, the non-fasted STB for these
 18 patients were > 52 μ mol/l (with the exception of one,

1 31 μ mol/l)^{10,12}. These non-fasted STB concentrations
2 already exceed the diagnostic range for GS¹⁴, hence
3 these patients have a more severe form of
4 hyperbilirubinaemia than those studied in this report,
5 while those in the Bosma et al¹² abstract had STB
6 concentrations similar to those studied here.

7
8 The example herein shows that the variation in the STB
9 levels after an overnight fast (and in the absence of
10 exposure to known inducers of the UGT1*1 isoform in GS,
11 such as alcoholic¹⁵ and drugs¹⁶) a representative group
12 of the Eastern Scottish population is primarily due to
13 (or associated with) the TATA box sequence variation
14 reported by Bosma et al¹². In agreement with previous
15 work females have a significantly lower mean STB
16 concentration than males¹⁷⁻¹⁸.

17
18 Individuals with the 7/7 genotype in the population
19 have GS (see Table 1). One of the 7/7 controls
20 indicated in Table 1 had a non-fasting STB similar to
21 those reported for heterozygous carriers of CN-2
22 mutations^{7,11} which suggests that this subject may also
23 be a carrier of a CN-2 mutation, alternatively, the
24 very elevated bilirubin in this patient may be due to
25 the coexistence of Reavon's Syndrome (characterized by
26 a collection of abnormal biochemical results which are
27 risk factors for coronary heart disease)¹⁹.

28
29 We have found that 10-13% of the Eastern Scottish
30 population have the genotype associated with mild GS.
31 None of the Gilbert subjects from the control
32 population were aware that they had an underlying
33 metabolic defect in glucuronidation with testifies to
34 its benign nature. Three 7/7 controls had STB
35 concentrations comparable to mean levels observed in
36 heterozygotes, however, they also had a lower than

1 normal PSAT ($\leq 22\%$) (see Figure 2). The observed
2 correlation between STB and PSAT ($p = 0.001$) (Figure 2)
3 and STB and iron (females $p = 0.001$ and males $p = 0.01$
4 including control h) indicates that other genetic and
5 environmental factors affecting the serum PSAT and iron
6 values will in turn affect the STB concentration.

7
8 From the data presented here and previous reports it
9 seems clear that there are mild and more severe forms
10 of GS. The milder form (fasted STB $25-50\mu\text{mol/l}$) is
11 either caused by (or is associated with) a homozygous
12 2bp insertion at the TATA sequence upstream of the
13 UGT1*1 exon 1 (autosomal recessive inheritance) while
14 the rarer more severe dominantly inherited forms
15 identified to date⁷⁻¹¹ (non-fasted STB $> 50\mu\text{mol/l}$) are due
16 to heterozygosity for a mutation in the coding region
17 of the UGT1*1 gene which in its homozygous state causes
18 CN-2. The particular genetic abnormality causing GS in
19 a patient will have implications for genetic
20 counselling as the dominantly inherited form of two GS
21 patients could result in offspring with CN-2, whereas
22 the recessive form in one or both GS patients would
23 have less serious implications. It is important to
24 discriminate between the two forms and provide suitable
25 genetic counselling for such couples. The rapid DNA
26 test presented here (less than 1 day for extracted DNA)
27 carried out in addition to biochemical tests following
28 a 12 hour overnight fast (without prior alcohol or drug
29 intake would permit such a diagnosis. The compliance
30 rate for the current 24 and 48 hour restricted diet
31 tests for GS¹³⁻¹⁴ is debatable and hence the overnight
32 fast has obvious advantages and only one blood sample
33 or a buccal smear is required (for genetic and
34 biochemical analysis) in contrast to the 2-3 blood
35 samplings required for the 24 and 48 hour tests. This
36 approach to GS testing would be cost effective in terms

1 of fewer patient return visits to clinics and in
2 identifying couples at risk of having children with
3 CN-2.

4
5 In addition, the recent finding of an increased
6 bioactivation of acetaminophen (a commonly used
7 analgesic which is eliminated primarily by
8 glucuronidation) in GS patients indicates the greater
9 potential for drug toxicity in these patients if
10 administered drugs which are also conjugated by UGT1
11 isoforms³. In fact, ethinylestradiol (EE2) has recently
12 been shown to be primarily glucuronidated by the UGT1*1
13 isoform in man²⁰ and hence this could have implications
14 for female Gilbert patients taking the oral
15 contraceptive who are then more predisposed to
16 developing jaundice.

17
18
19 The tests outlined herein have obvious implications for
20 setting up drug trials in understanding unusual results
21 in ruling out individuals who may be adversely affected
22 by the drugs or in positively choosing these
23 individuals to determine the effects of particular
24 drugs on hyperbilirubinaemia.
25

1 References

- 2
- 3 1 Fevery, J. Pathogenesis of Gilbert Syndrome. *Eur.*
4 *J. Clin. Invest.* 1981;11; 417-418.
- 5
- 6 2. Watson, K.J.R. and Gollan, J.L. Gilbert's
7 Syndrome. *Bailliere's Clinical Gastroenterology*
8 1989; 3: 337-355.
- 9
- 10 3. De Morais, S.M.F., Uetrecht, J.P. and Wells, P.G.
11 Decreased glucuronidation and increased
12 bioactivation of acetaminophen in Gilbert's
13 Syndrome. *Gastroenterology* 1992; 102: 577-586.
- 14
- 15 4. Carulli, N., Ponz de Leon, M., Mauro, E., Manenti,
16 F and Ferrari, A. Alteration of drug metabolism in
17 Gilbert's Syndrome. *Gut* 1976; 17: 581-587.
- 18
- 19 5. Macklon, A.F., Savage, R.L. and Rawlins, M.D.
20 Gilbert Syndrome and drug metabolism. *Clin.*
21 *Pharmacokinetics* 1979; 4: 223-232.
- 22
- 23 6. Thompson, R.P.H. Genetic transmission of
24 Gilbert's Syndrome in "Familial
25 Hyperbilirubinaemia", (Ed. L. Okoliosanyi), *John*
26 *Wiley & Sons Ltd*; 91-97.
- 27
- 28 7. Gollan, J.L. Huang, S.N., Billing, B. and
29 Sherlock, S. Prolonged survival in three brothers
30 with severe type 2 Crigler-Najjar Syndrome.
31 *Gastroenterology* 1975; 68: 1543-1555.
- 32
- 33 8. Moghrabi, N., Clarke, D.J., Boxer, M. and
34 Burchell, B. Identification of an A-to-G missence
35 mutation in exon 2 of the UGT1 gene complex that
36 causes Crigler-Najjar Syndrome type 2. *Genomics*

- 1 1993; 18: 171-173.
2
- 3 9. Moghrabi, N.N. Molecular Genetic Analysis of the
4 Human Phenol and Bilirubin UDP-
5 Glucuronosyltransferase Gene Complex and
6 Associated Disease Syndromes. *PhD thesis* 1994,
7 University of Dundee, Dundee, Scotland.
8
- 9 10. Aono, S., Adachi, Y., Uyama, E., Yamada, Y.,
10 Keino, H., Nanno, T., Koiwai, O. and Sato, H.
11 Analysis of genes for bilirubin UDP-
12 glucuronosyltransferase in Gilbert's Syndrome,
13 *Lancet* 1995; 345: 958-959.
14
- 15 11. Koiwai, O., Nishizawa, M., Hasada, K., Aono, S.,
16 Adachi, Y., Mamiya, N. and Sato, H. Koiwai, O.,
17 Nishizawa, M., Hasada, K., Aono, S., Adachi, Y.,
18 Mamiya, N. and Sato, H. Gilbert's Syndrome is
19 caused by a heterozygous missence mutation in the
20 gene for bilirubin UDP-glucuronosyltransferase.
21 *Hum. Molec. Genet.* 1995; 4: 1183-1186.
22
- 23 12. Bosma, P., Goldhoorn, B., Bakker, C., Out, T., Roy
24 Chowdhury, J., Roy Chowdhury, N., Oostra, B.,
25 Lindhout, D., Michiels, J., Jansen, P., Tytgat, G.
26 and Oude Elferink, R. Presence of an additional TA
27 in the TATAA box of B- UGT1 correlates with
28 Gilbert Syndrome. *Hepatology* October 1994;
29 Abstract 680: 226A.
30
- 31 13. Owens, D. and Sherlock, S. Diagnosis of Gilbert's
32 Syndrome: role of reduced calorie intake test.
33 *Br. Med.J.* 1973; 3: 559-563.
34
- 35 14. Lascelles, P.T. and Donaldson, D. Calorie
36 restriction test in "Diagnostic Function Tests in

- 1 Chemical Pathology" Kluwer Academic Publishers
2 1989: 24-25.
3
- 4 15. Ideo, G., De Franchis, R., Del Ninno, E. and
5 Dioguardi, N. Ethanol increases liver uridine-
6 diphosphate-glucuronosyltransferase. *Experientia*
7 1971; 27: 24-25.
8
- 9 16. Sutherland, L.T., Ebner, T. and Burchell, B.
10 Expression of UDP-Glucuronosyltransferases (UGT) 1
11 family in human liver and kidney. *Biochem.*
12 *Pharmacol.* 1993; 45: 295-301.
13
- 14 17. Owens, D. and Evans, J. Population studies on
15 Gilbert Syndrome. *J. Med. Genet.* 1975;12: 152-
16 156.
17
- 18 18. Bailey, A., Robinson, D. and Dawson, A.M. Does
19 Gilbert's disease? *Lancet* 1977; 1: 931-933.
20
- 21 19. Reaven, G.M. Syndrome X: 6 years later. *J.*
22 *Intern. Med.* 1994; 236: 13-22.
23
- 24 20. Ebner. T., Remmel, R.P. and Burchell, B. Human
25 bilirubin UDP-glucuronosyltransferase catalyses
26 the glucuronidation of ethinylestradiol. *Molec.*
27 *Pharmacol.* 1993; 43: 649-654.
28
- 29 21. Edwards, A., Hammond, H.A., Jin, L., Caskey, C.T.
30 and Chakraborty, R. Genetic variation at five
31 trimeric and tetrameric tandem repeat loci in four
32 human population groups. *Genomics* 1992; 12: 241-
33 253.

1 **CLAIMS**

2

3 1. A method for improving the efficacy of drug
4 trials, the method comprising the step of
5 screening samples from potential participants for
6 the genetic basis of Gilbert's Syndrome and
7 eliminating or including potential participants in
8 a drug trial in the knowledge of them possessing
9 or not possessing the genetic basis of Gilbert's
10 Syndrome.

11

12 2. A method as claimed in claim 1 comprising the
13 steps of:

14

15 a) taking a sample from each potential
16 participant in a drug trial,

17

18 b) screening the samples for the genetic basis
19 of Gilbert's Syndrome,

20

21 c) identifying participants having the genetic
22 basis of Gilbert's Syndrome, and

23

24 d) proceeding with drugs trials in the knowledge
25 of participants possessing or not possessing
26 the genetic basis of Gilbert's Syndrome.

27

28 3 A method as claimed in claim 1 or 2 wherein the
29 sample is chosen from blood, buccal smear or any
30 other sample containing DNA from the potential
31 participants.

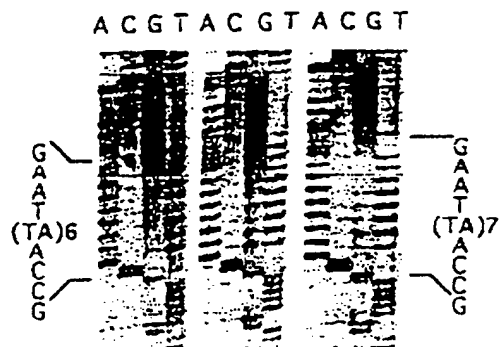
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33 4. A method as claimed in any of the preceding claims
34 further comprising the step of eliminating
35 participants having the genetic basis of Gilbert's
36 Syndrome from a drugs trial.

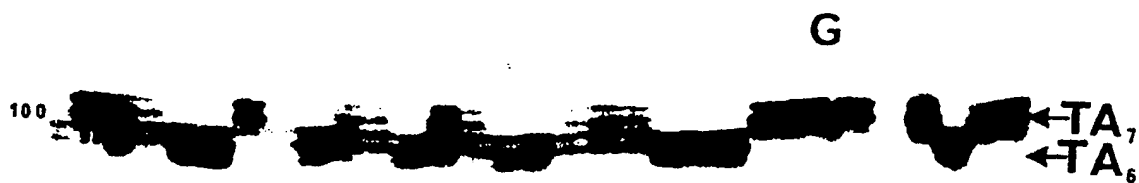
- 1 5. A method as claimed in any of claims 1 to 3
2 wherein the method comprises the further step of
3 selecting only participants having genetic basis
4 for Gilbert's Syndrome for a drugs trial.
5
- 6 6. A method as claimed in any of claims 1 to 3
7 further comprising the step of interpreting the
8 results of the drugs trial in the knowledge that
9 certain participants have Gilbert's Syndrome.
10
- 11 7. A method as claimed in any of the preceding claims
12 wherein the method comprises the steps of:
13
- 14 a) isolating DNA from each sample,
15
- 16 b) amplifying the DNA inner region indicating
17 the genetic basis for Gilbert's Syndrome,
18
- 19 c) isolating amplified DNA fragments, and
20
- 21 d) identifying individuals having the genetic
22 basis of Gilbert's Syndrome.
23
- 24 8. A method as claimed in any of the preceding claims
25 wherein the DNA is amplified using the polymerase
26 chain reaction (PCR) using a radioactively
27 labelled pair of nucleotide primers.
28
- 29 10. A method as claimed in any of claims 7 to 9
30 wherein the DNA region indicating the genetic
31 basis of Gilbert's Syndrome is the gene encoding
32 UDP-glucuronosyltransferase (UGT).
33
- 34 11. A method as claimed in any of claims 7 to 10
35 wherein the DNA to be amplified is in an upstream
36 promoter region of the UGT 1*1 exon 1.

- 1 12. A method as claimed in any of claims 7 to 11
2 wherein the DNA to be amplified includes the
3 regions between -35 and -55 nucleotides at the 5'
4 end of UGT 1*1 exon.
5
- 6 13. A kit for screening individuals participation in
7 drug trials, the kit comprising primers for
8 amplifying DNA in the region of the genome
9 indicating the genetic basis of Gilbert's
10 Syndrome.
11
- 12 14. Primers for use in a method as claimed in any of
13 the preceding claims including primer pairs, AB or
14 CD as follows:
15
- 16 A/B (A, 5'-AAGTGAAGTCCCTGCTACCTT-3',
17 B, 5'-CCACTGGGATCAACAGTATCT-3') or
18 C/D (C, 5'-GTCACGTGACACAGTCAAAC-3';
19 D 5'-TTTGCTCCTGCCAGAGGTT-3').

1/4

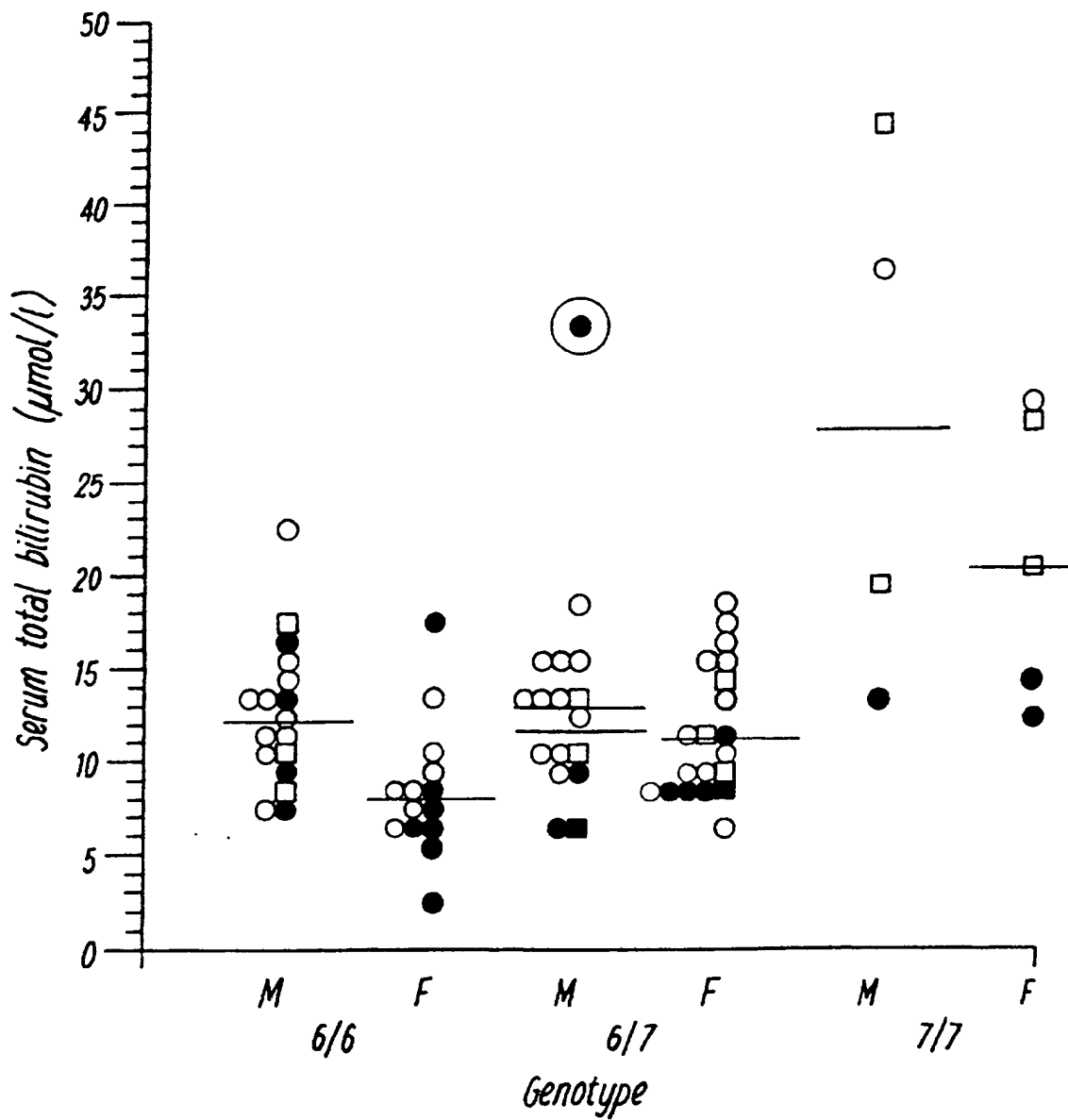


FrE 1a

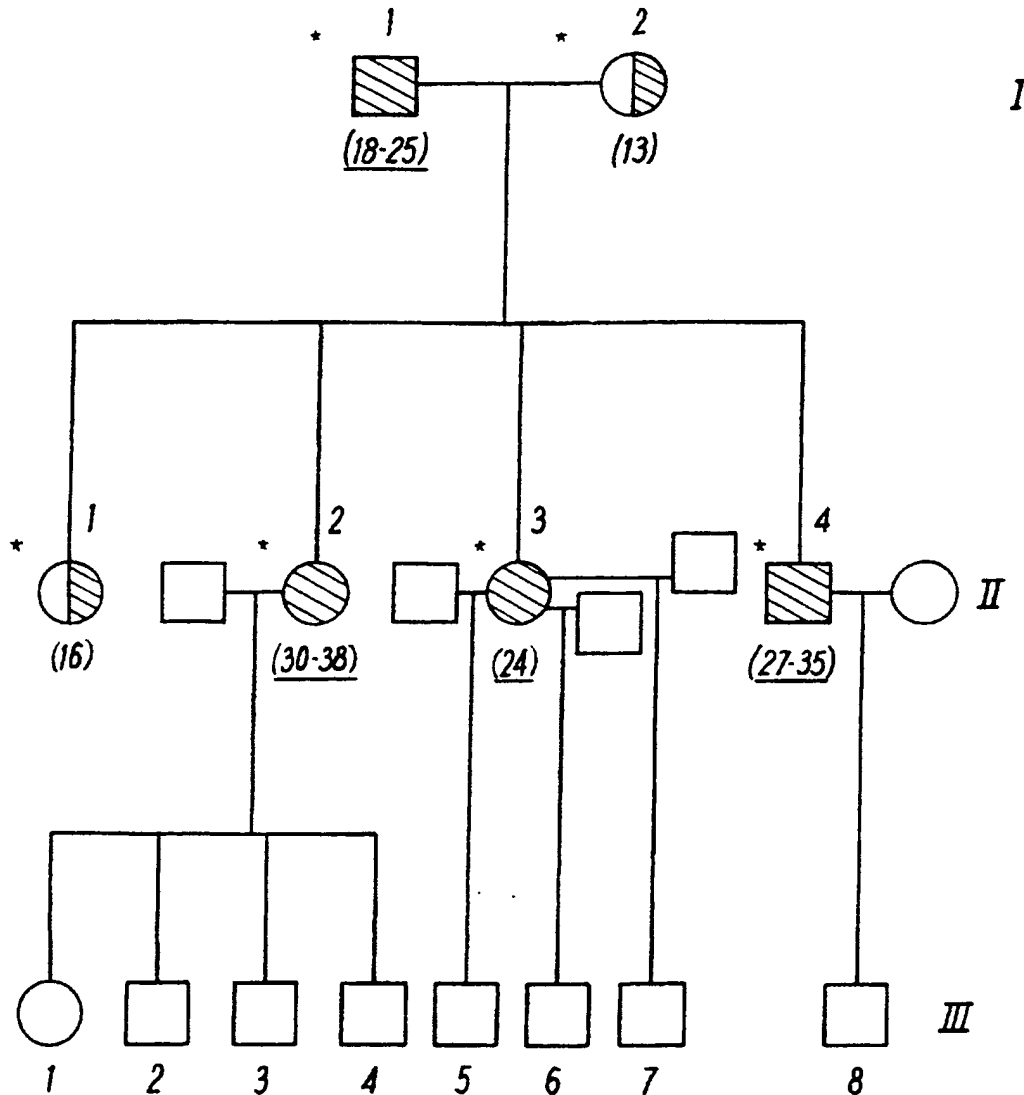


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2/4

**FIG. 2**

Pedigree Showing Segregation of the Gilbert Phenotype with $\frac{3}{4}$ the $(TA)_7TAA / (TA)_7TAA$ Genotype.



*I, II, III = generations in family * = genetic and biochemical data available*

☐ male

☐ female

☒ ☒ homozygotes for the $(TA)_7TAA$ allele

☒ heterozygotes for the $(TA)_7TAA$ and $(TA)_6TAA$ alleles

(13) = total serum bilirubin

(18-25) = elevated total serum bilirubin

Fig. 3

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4/4

AP1

-611 GTGAGTCTGGCTCACCTCATGGCGCTGGCTCGTGTGGTGGGCTCTGCTGCAGCCTCCAA

Sp1

-541 GACACCACACTGTGCTG6ACTCAATAAATAATGTTGGACGAAGGAATGAAACACATGATA

-491 CAAGTGAGCAGGCAGTACCGGGGGAGCTGTGGAGTGGGCACTCTTACAGGTTTCCATGGC

-431 GAAAGCGGGGGACAGTTGTGTTCTTTTCTTTCTAAAAGGCTTTCTAAAAAGCCTTCTGT

-371 TTAATTTCTGGAAAAGAAGCCTAACTTGTTCACTACATAGTCGTCCTTCTTCCCTCTCTGG

AP3

-311 TAACACTTGTTGGTCTGTGGAAATACTAATTTAATGGATCCTGAGGTTCTGGAAGTACTT

CLBP

AP1

-251 TGCTGTGTTCACTCAAGAATGTGATTGAGTATGAAATTCAGCCAGTTCAACTGTTGTT

-191 GCCTATTAAGAAACCTAATAAAGCTCCACCTTCTTTATCTCTGAAAAGTGAACCTCCCTGCT

AP1

HNF1

-131 ACCTTTGTG6ACTGACAGCTTTTTATAGTCACGTGACACAGTCAAACATTAACTTGGTGT

-71 ATCGATTGGTTTTTGCCATATATATATATAAGTAGGAGAGGGCGAACCTCTGGCAGGA

-11 GCAAAGGCGCCATGGCTGTG

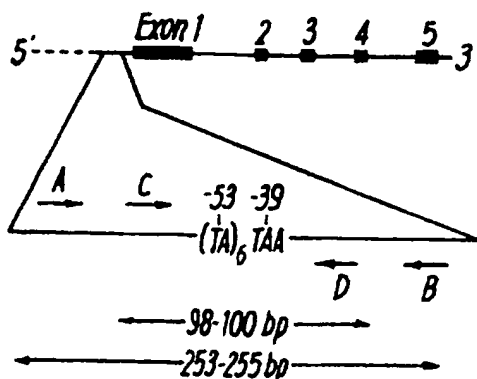


Fig. 4

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